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Supporting Information

Diketopiperazine Formation in Fungi Requires Dedicated Cyclization and Thiolation Domains

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1 Supporting Methods

1.1 Strains, media, and growth conditions

The fungal strains used in this study are listed in **Table S3**. Unless otherwise noted, all strains were grown at 30 °C on glucose minimal medium (GMM[1]) and, when appropriate, were supplemented with 0.56 g uracil L^{-1}, 1.26 g uridine L^{-1}, 1.0 g arginine L^{-1} and maintained as glycerol stocks at -80 ºC. *Escherichia coli* strain DH5α was propagated in LB medium with appropriate antibiotics for plasmid DNA.

1.2 Gene cloning, plasmid construction, and genetic manipulations

(a) *A. fumigatus* GliP truncation and complement strains: The *gliP* C_TT_3 or T_3 domain deletion strain (TJW139 or TJW140 respectively) was created in strain Af293.1 by replacing the C_TT_3 or T_3 domain with *A. fumigatus pyrG* using modified double joint PCR[2] consisting of the following: 1 kb DNA fragment upstream of the C_TT_3 or T_3 domain, a 1.9 kb DNA fragment of *A. fumigatus pyrG* with glutathione gene terminator (primers glutapyrGF and glutapyrGR),[1,3] and a 1 kb DNA fragment downstream of the C_TT_3 or T_3 domain. 30 µL of Sephadex® G-50 purified third round PCR product was used for fungal transformation. Polyethylene glycol based fungal transformation was done as previously described.[2,4] C_TT_3 or T_3 domain deletants were confirmed by PCR and Southern blot (**Figure S11a**) and the correct transformants, TJW139.30 and TJW140.16, were used for subsequent analysis.

For ΔC_TT_3 complementation, pJW162 was created by inserting a 3.6 kb PCR product with *gpdA* promoter using the primer pair gliPgpdF/CTglipR and cloning the subsequent product into *BamH*I/*Hind*III sites of pUCH2-8.[5] This plasmid was used to transform TJW139.30 to complement a deletion of ΔC_TT_3. The resulting strain was called TJW178 and was confirmed by PCR and Southern blotting (**Figure S11b**). All fungal strains used in this study are listed in **Table S3**, and primers are listed in **Table S4**.

(b) *A. fumigatus* GliP point mutation: To introduce the histidine-to-alanine amino acid substitution in *A. fumigatus*, we first fully deleted *gliP* in a strain with a deleted *akuA* gene (TFYL44.1) to increase the rate of homologous recombination and decrease the amount of transformants that need to be screened to obtain the desired strain. The open reading frame of *gliP* was replaced with a copy of *pyrG* from *A. parasiticus* to complement the *pyrG* auxotrophy. To generate a construct to delete *gliP*, the flanking regions of the *gliP* open reading frame were amplified (gliP3'-F & gliP3' R, and gliP5'-F & gliP5'-R) as well as the *A. parasiticus pyrG* gene (Ap-pyrGF & Ap-pyrGR). These PCR products were fused using double joint PCR and used to transform TFYL44.1 to create strain TBTP12.02 which was confirmed by Southern blot analysis (**Figure S12**).[6] Two plasmids were then generated, one which included a full length copy of *gliP* (pBTP12), and one that contained a H1754A copy of *gliP* (pBTP13), both targeted to the *akuA* locus. The pBTP12 plasmid was assembled by amplifying *akuA* flanks (KU5'-F & KU5'-R, and KU3'-F & KU 3'-R), *gliP* (gliP-F & gliP-R), and

A. fumigatus argB as the selectable marker (AFU argB fwd & AFU argB rev). These PCR fragments were combined with a plasmid backbone amplified from a yeast shuttle vector (YS F/YS R) in a yeast transformation to allow for homologous recombination to assemble the fragments into a full plasmid. pBTP13 was assembled using the same fragments and method, except that the H1754A substitution was introduced by using primers containing the mutation and amplifying *gliP* in two separate PCR reactions (gliP-F & gliP-H1754A-R, and gliP-H1754A-F & gliP-R). TBTP12.02 was then transformed with pBTP12 and pBTP13 to generate TBTP99 and TBTP100 respectively, which were confirmed by Southern blot analysis (**Figure S13**). TBTP12.02 was taken to prototrophy by amplifying *argB* (AFU argB fwd &AFU argB rev2) from *A. fumigatus* and selecting prototrophic transformants generating TBTP94.

(c) Heterologous *gliP* expression vectors: pET24b GliP was a gift from Robert A. Cramer, Jr. (Durham, NC), which was constructed as described.[7] Truncations were made by PCR and reinstalled into pET24b with the previously utilized NdeI/XhoI restriction sites. All *gliP* mutants were constructed by applying PCR site-directed mutagenesis on the original pET24 *gliP* as template using primers listed in **Table S4**. Competent *E. coli* NEB® 5-alpha (New England Biolabs) was transformed with the PCR reactions, which were sequenced to confirm accurate amplification.

(d) *A. fumigatus* GliP point mutation at amino acid 2095 (ser->ala) : To replace serine (**T**CG) to alanine (**G**CG) at 2095 amino acid position, we first created a single point mutation (T→G) using jont PCR. This mutated template was fused to a 1.9 kb DNA fragment of *A. fumigatus pyrG* with glutathione gene terminator (primers DgPT5'F and DgPCT3R) by joint PCR[1,3]. The fused 3kb PCR amplicon with the point mutation was used for transformation to Af293.1. Transformants were confirmed by Southern blotting (**Figure S14**) and sequeincing (data not shown) to obtain TJW201.38 for the subsequent experiments.

1.3 Nucleic acid analysis

Plasmid preparation, digestion with restriction enzyme, gel electrophoresis, blotting, hybridization, and probe preparation were performed by standard methods.[8] *Aspergillus* DNA for diagnostic PCR was isolated using the previously described method.[9] Sequence data were analyzed using the LASERGENE software package from DNASTAR.

1.4 Northern analysis

Strains were grown in liquid GMM at a concentration of 1.0×10^6 spores per milliliter shaking at 225 rpm at 30 ºC for 24 hours, then 25 ºC for an additional 48 h or after 24 Gliotoxin (**1**), was added at 25 µg/mL followed

by an additional 24 h of cultivation. Mycelia were harvested by filtering through Miracloth (CalBioChem), lyophilized, and total RNA was then isolated using Trizol (Invitrogen). The probe for *gliG* was prepared by PCR amplification of genomic DNA, and labeled with dCTP αP.[32]

1.5 Fermentation and metabolome extraction

A. fumigatus strains were inoculated (1.0×10^6 spores/mL) into 25 mL GMM in 125 mL Erlenmeyer flasks at 30 °C with shaking at 220 rpm. After 5 days, liquid fungal cultures including fungal tissue and media were frozen using a dry ice-acetone bath and lyophilized. The lyophilized residues were extracted with 12.5 mL of a mixture of acetonitrile, ethyl acetate, and water (80:15:5) for 0.5 h with vigorously stirring. Extracts were filtered over cotton, evaporated to dryness, and stored in 8 mL vials. Crude extracts were suspended in 1.0 mL of extraction solvent and centrifuged to remove insoluble materials, and the supernatant was subjected to LC-HRMS analysis.

1.6 Analytical methods and equipment overview

(a) NMR spectroscopy: NMR spectroscopic instrumentation: a Bruker Avance^{III} HD (800 MHz ^{1}H reference frequency, 201 MHz for ^{13}C) equipped with a 5 mm CPTCL ^{1}H-^{13}C/^{15}N cryo probe. Non-gradient phase-cycled dqfCOSY spectra were acquired using the following parameters: 0.6 s acquisition time, 400-600 complex increments, 8, 16 or 32 scans per increment. Non-gradient HSQC, HMQC, and HMBC spectra were acquired with these parameters: 0.25 s acquisition time, 200-500 complex increments, 8-64 scans per increment. ^{1}H, ^{13}C-HMBC spectra were optimized for $J_{H,C}$ = 6 Hz. HSQC spectra were usually acquired without decoupling. NMR spectra were processed and baseline corrected using MestreLabs MNOVA software packages. (b) Mass spectrometry: LC-HRMS was performed on a Thermo Scientific-Dionex Ultimate3000 UHPLC system equipped with a diode array detector and connected to a Thermo Scientific Q-Exactive Orbitrap operated in electrospray positive (ESI^+) or electrospray negative (ESI^-) ionization mode. Low-resolution HPLC-MS was performed on an Agilent 1100 series HPLC system equipped with a diode array detector and connected to a Quattro II mass spectrometer (Micromass/Waters) operated in ESI^+ or ESI^- mode. Data acquisition and processing for the LC-HRMS was controlled by Thermo Scientific Xcalibur software. Data acquisition and processing for the HPLC-MS was controlled by Waters MassLynx software. (c) Chromatography: flash chromatography was performed using a Teledyne ISCO CombiFlash system. For

semi-preparative HPLC Agilent Zorbax Eclipse XDB-C18 or -C8 columns (25 cm x 10 mm, 5 µm particle diameter) were used. An Agilent Zorbax Eclipse XDB-C18 column (4.6 x 250 mm, 5 µm particle diameter) was used in the HPLC-MS analyses of *in vitro* protein activity assays. For semi-preparative and analytical HPLC acetonitrile (organic phase) and 0.1 % acetic acid in water (aqueous phase) were used as solvents at a flow rate of 3.20 mL/min or 1.0 mL/min, respectively. A solvent gradient scheme was used, starting at 5% organic for 3 min, followed by a linear increase to 100% organic over 25 min, holding at 100% organic for 8 min, then decreasing back to 5% organic for 1 min and holding at 5% organic for the final 6 min, a total of 40 min. An Agilent Zorbax RRHD Eclipse XDB-C18 column (2.1 x 100 mm, 1.8 µm particle diameter) heated to 40 °C was used in the LC-HRMS *A. fumigatus* mutant profiling analysis with acetonitrile (organic phase) and water (aqueous phase) with 0.1 % acetic acid used as solvents at a flow rate of 0.5 mL/min. For data displayed in Figure 2 a solvent gradient scheme was used, starting at 5% organic with an immediate linear increase to 100% organic over 10.5 min, holding at 100% organic for 4 min, then decreasing back to 5% organic in 0.1 min and holding for the final 1.5 min, for a total of 16 min. For data displayed in Figure 4 a solvent gradient scheme was used, starting at 5% organic for 5 min, then a linear increase to 100% organic over 15 min, holding at 100% organic for 5 min, then decreasing back to 5% organic in 0.1 min and holding for the final 2.9 min, for a total of 28 min.

1.7 Heterologous protein production

All C-terminal hexahistidine-tagged GliP mutants, and truncation expression constructs were used to transform *E. coli* BL21(DE3) (New England Biolabs), which was grown in Terrific Broth (TB) supplemented with 10 mM $MgCl_2$ and selected with 100 µg/mL ampicillin. 10 mL overnight cultures were diluted into 1 L of TB in a 4 L flask and shaken at 200 RPM at 37 °C to an OD of approximately 0.75, cooled to 16 °C and further grown to an OD of roughly 1.0-1.2 and induced with 100 µM IPTG. Cultures were maintained at 16 °C at 200 RPM for an additional 24 hours before harvesting at 5,000 x g (4 °C for 10 min) and stored at -80 °C until purification. All further steps occurred at 4 °C unless otherwise noted. 20 g of frozen pellets were resuspended in 150 mL of 25 mM Tris pH 8.0, 500 mM NaCl, and sonicated. Lysed cells were spun at 20,000 x g for 20 min, and the supernatant was collected and gently stirred with 1 mL HisPur Ni-NTA Resin (Thermo Fisher Scientific) for 30 min. During incubation, 5 µL of Benzonase (EMD Millipore) was added along with 1 mM $MgCl_2$. The slurry was loaded and passed through a column and the resin was washed with 20 column volumes of fresh lysis buffer. The protein was then eluted with 30 mL lysis buffer containing 150 mM imidazole and 10% glycerol. The elution was concentrated with an Amicon Ultra-15 30 kDa spin filter (EMD Millipore), flash frozen over liquid nitrogen and stored at -80 °C until further purification. FPLC purification of proteins were performed using a HiLoad 16/600 Superdex 200 preparatory grade column run

on a Amersham Biosciences P-920 pump equipped with a UPC-900 detector and a Frac-950 fraction collector (GE Healthcare) with a running buffer of 20 mM Tris, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, and 1 mM DTT. Fractions containing the protein of interest were combined and concentrated with an Amicon Ultra-15 30 kDa spin filter and flash frozen over liquid nitrogen and stored at -80 °C until further analysis was required.

1.8 GliP product formations assays

GliP assays were first pantetheinylated in 75 mM Tris pH 8.0, 5 mM $MgCl_2$, 300 µM coenzyme A, 1 µM Sfp synthase (New England Biolabs), and 1 µM GliP (or GliP mutant) in 100 µL reactions at 25 ºC for 1 hour. Following Sfp incubation, an additional 100 µL solution of 10 mM ATP, 800 µM phenylalanine, and 800 µM serine was added to initiate catalysis. Reactions were monitored with low-resolution HPLC-MS, as described above.

1.9 GliP T_3-pantetheine detection

1 µM GliP in 100 µL was incubated with 75 mM Tris pH 8.0, 5 mM $MgCl_2$, 300 µM coenzyme A, 1 µM Sfp synthase at 25 °C. After 1 hour, proteins were digested with 1.5 µg of sequencing grade chymotrypsin (Promega) for 12 hours. Peptides were then reduced with 5 mM DTT for 20 minutes at 50 ºC, and thiols were capped with 15 mM iodoacetamide. Formic acid was added to 1 % (v/v), and peptides were prepared with 100-µl Pierce C18 Tips (Thermo Scientific) per the manufacturer's protocol using 100 µL of the elution solution. Peptides were identified via LC-HRMS, as described above.

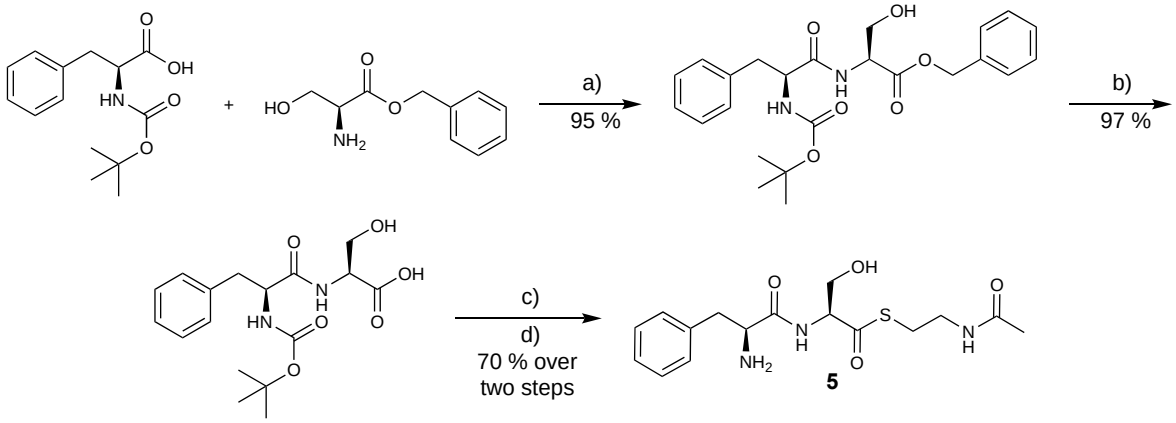
1.10 Compound 5 cyclization assay

1 µM GliP_S555A_S1582A in 100 µL was incubated with 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 100 µM coenzyme A, 1 µM Sfp synthase at 25 °C for 30 min. Catalysis was initiated with 1 µL of 100 mM **5**, and after 10 minutes the reaction was quenched with 100 µL acetonitrile and immediately frozen over liquid nitrogen. Samples were thawed only immediately before HPLC analysis. Analysis was performed with low-resolution HPLC-MS, as described above.

1.11 ATP-[^{32}P]pyrophosphate exchange assays

The reactions were set up in a total assay volume of 100 µL at 25 °C in 100 mM phosphate buffer, 5 mM $MgCl_2$, 125 nM EDTA, 5 mM ATP, 100 nM purified GliP proteins, 0.1 µM [^{32}P]pyrophosphate (50 Ci/mmol), and 1 mM amino acid substrate were added. The reaction proceeded for 30 min before it was stopped (1 % (w/v) activated charcoal, 4.5 % (w/v) tetrasodium pyrophosphate, 3.5 % (v/v) perchloric acid) and further processed as described.[10] Pyrophosphate exchange was quantified on a scintillation counter (PerkinElmer TriCarb 2910TR).

1.12 Synthesis of *N*-acetylcystamine-L-Phe-L-Ser (5)



a) EDC (1.2 eq), HOBt (1.2 eq.), DIEA (3 eq.) DMF, 24 h. b) H_2 (continuous stream), Pd/C (10 mol %), 1.5 h. c) *N*-acetylcystamine (1.5 eq.), EDC (1.2 eq.), HOBt (1.2 eq.), DIEA (3 eq.) DMF, 24 h. d) 40 % TFA, DCM, 1 h.[11] e) 1 M phosphate buffer, pH 8, 48 h. See below for NMR assignments and spectra of **5.**[12]

1.13 Mining for putative DKP producing NRPSs with ATCATC$_T$T$_C$ domain architecture (Table S1).

GliP (accession: AAW03307.1) was used as a query sequence for a blastp search of NCBI's Fungi (taxid: 4751) non-redundant protein database using the default parameters with a total of 20000 subject sequences. The resulting hits were exported to an excel sheet and dereplicated (for multiple alignments to the same sequence) and sorted for size (2100-2300 amino acids) to obtain NRPSs with the correct domain architecture. The resulting sequences (156) were parsed with Python to search for the conserved residues "SHXXXDXXS/T" in the C_T active site, which yielded 89 sequences that were manually curated to ensure

correct domain architecture and to remove homologs >95 % similarity to GliP, which likely produce gliotoxin. The resulting 56 putative NRPSs are annotated in **Table S1**.

1.14 Gene sequences for GliP-WT and mutant GliP proteins.

GliP – WT (AFUA_6G09660)

ATGCCATCAGTAGTAGCGCTCGACCTCTGCCAGCTTTTTGACCGGTCCGTCGCTCGGACACCACACC
AGCTGGCAGTCGATCATGAGAGCGGCTCGCTCACCTATACCGAACTCGATGTGGCCTCATCGAACCT
GGCCCGAAAGCTAAAGCAAGAAGGAGTAGTCCCTGGGGAAGCGGTCCTCTTACTTACTGAGCACGG
CACCCGGAATGTTGTCGCGCTGCTTGCCATCCTCAAGGCCCACGCCTGCTACGTTCCTCTGGACCGC
AGCTCGTGGTCATCAGAGCGGATCCAGGCCGTGCTGGACGGGACAGACAGCCGGATTCTGATCAAC
ACAACCGTCGAGCCGTTCGAAAGCCCGCGGCACAAAGTCATCCATCTGACCAGCGCCGATGTGACG
ACTCTATCGACGGACCGCAGCACCACAAAGGTCATTCCCGACATCGCTCCCGAAGACCTCGCTTGTT
TGATCTTTACCAGTGGGAGCACAGGTGTGCCCAAGGGAGTCATGATTCCACATCGTGCCGTAGCCAA
TTATGCTCAGACCAGTCCATTCAACATGGATGTGCAGCCGGGAGACCGGGTACTGCATATCCTGTCG
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CTTTCCATCGGCCGGGACGATACTTGGCTGTCGATCAGTTTCCACTGTCAGCCAACGGCAAGATTGA
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GliP-ΔT_3

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GliP-$\Delta C_T T_3$

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GliP-C_TT_3 only

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2 Supporting Figures

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Figure S1. Amino acid sequence of C_TT_3 domains of GliP (Af293). Residues highlighted in yellow are conserved across C_T domains.[13]

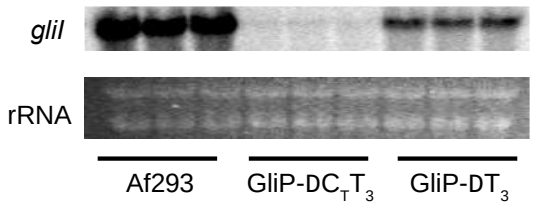


Figure S2. *gliI* gene expression in WT(Af293) GliP-ΔC_TT_3 and GliP-ΔT_3 *A. fumigatus* strains**.** 10^7 spores/mL were inoculated in 50 mL liquid GMM and incubated for 72 h at 25 ºC, 225 rpm, before total RNA extraction.

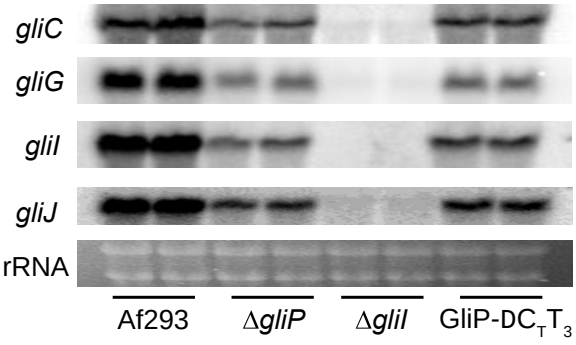


Figure S3. Gene expression of *gliC, gliG, gliI* and *gliJ* in WT(Af293), ΔGliP, ΔGliI, and GliP-ΔC_TT_3 *A. fumigatus* strains. 10^7 spores/mL were inoculated in 50 mL liquid GMM and incubated for 24 h at 30 ºC, 225 rpm and an additional 24 h at 25ºC, 225 rpm before adding gliotoxin (**1**, 25 mg/mL). After 24 h of further cultivation at 25 ºC, total RNA was isolated from samples for gene expression analysis.

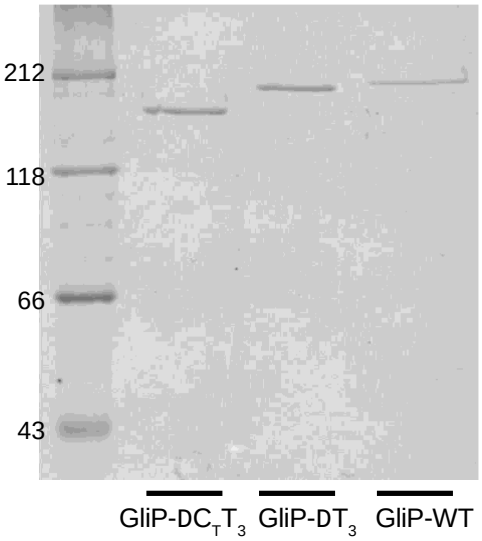


Figure S4. SDS-PAGE confirmation of recombinant GliP-$\Delta C_T T_3$, GliP-ΔT_3, and GliP-WT.

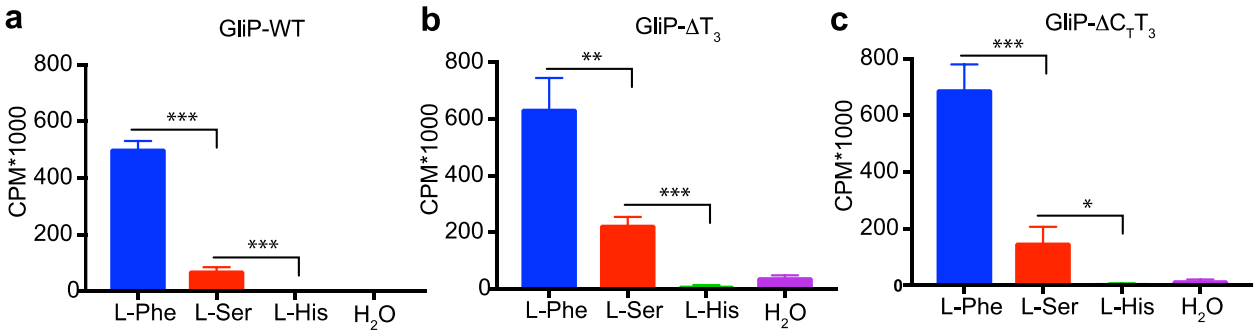


Figure S5. ATP-[^{32}P]PP$_i$- radioisotope exchange assay results for (a) recombinant GliP-WT, (b) GliP-ΔT_3, and (c) GliP-$\Delta C_T T_3$. Shown are raw turnover rates for L-Phe, L-Ser, L-His, and water for each enzyme,[14] each assay was run in triplicate and analyzed with a student's *t*-test, *P < 0.05, **P < 0.005, ***P < 0.0005.

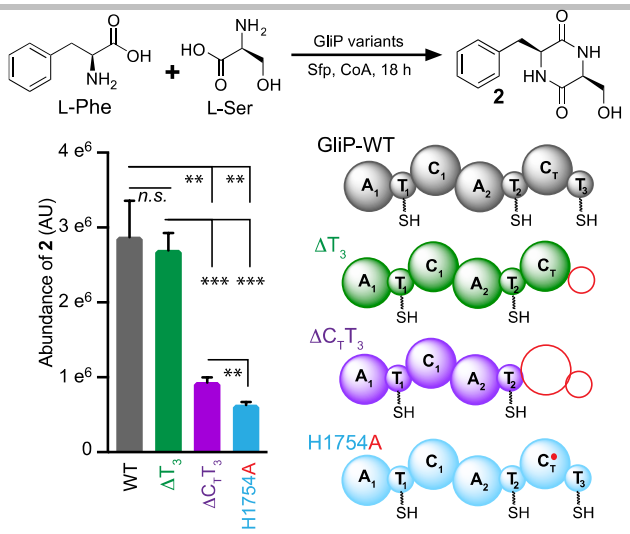


Figure S6. *In vitro* product formation assays with GliP. (top) L-Phe and L-Ser incubated with purified GliP variants furnishes **2**. (bottom) Quantification of relative yield of **2** from each assay, as measured by integration of LC-MS ion-chromatograms (n = 4). $^{**}p < 0.01$, $^{***}p < 0.001$.

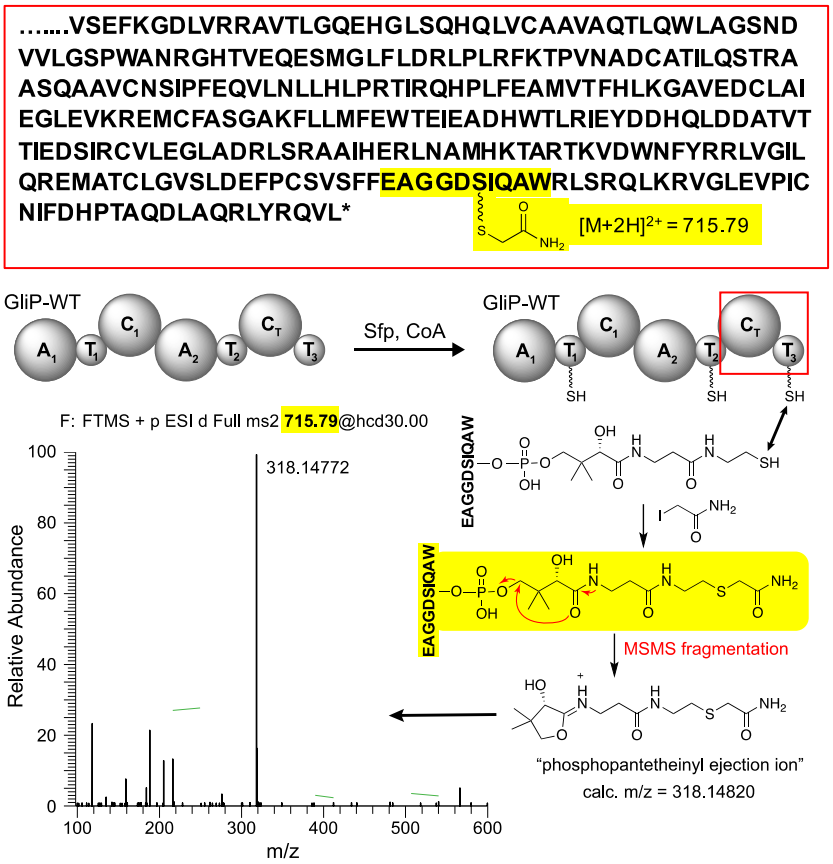


Figure S7. LC-HRMS/MS confirmation of phosphopantetheinyl modification of GliP-T_3. See Supporting Methods for experimental details.[15]

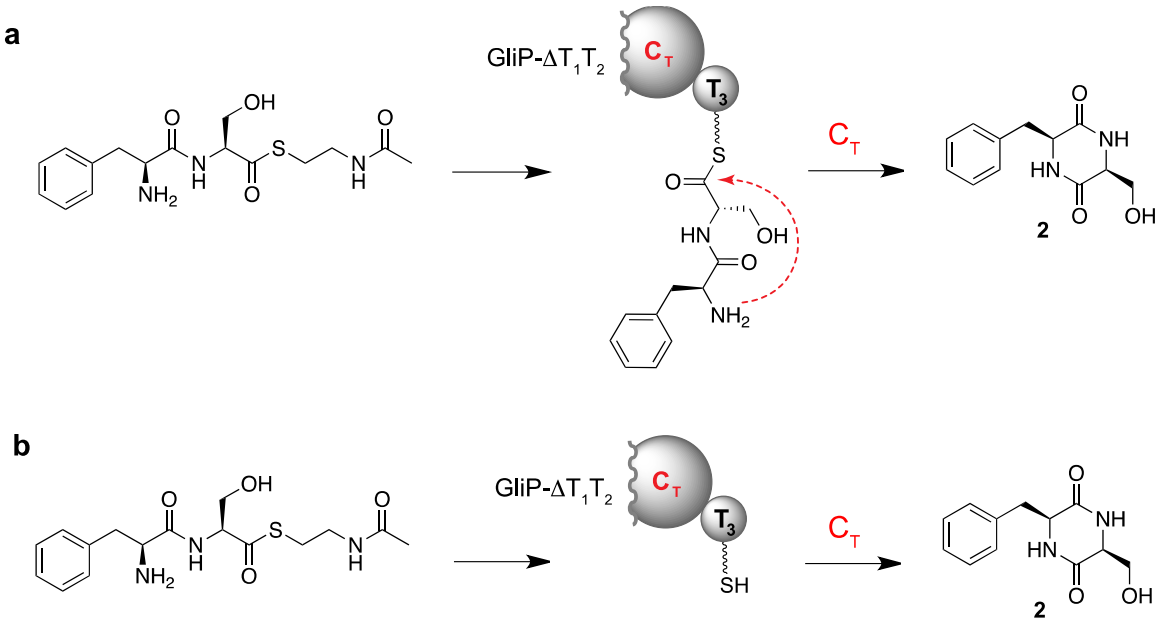


Figure S8. *In vitro* cyclization activity of GliP-ΔT_1T_2 toward **5.** (a) **5** can first be loaded onto T_3 via trans-thiolation, then cyclized by the C_T domain to form **2**, or (b) the C_T domain can directly cyclize **5** to form **2**.

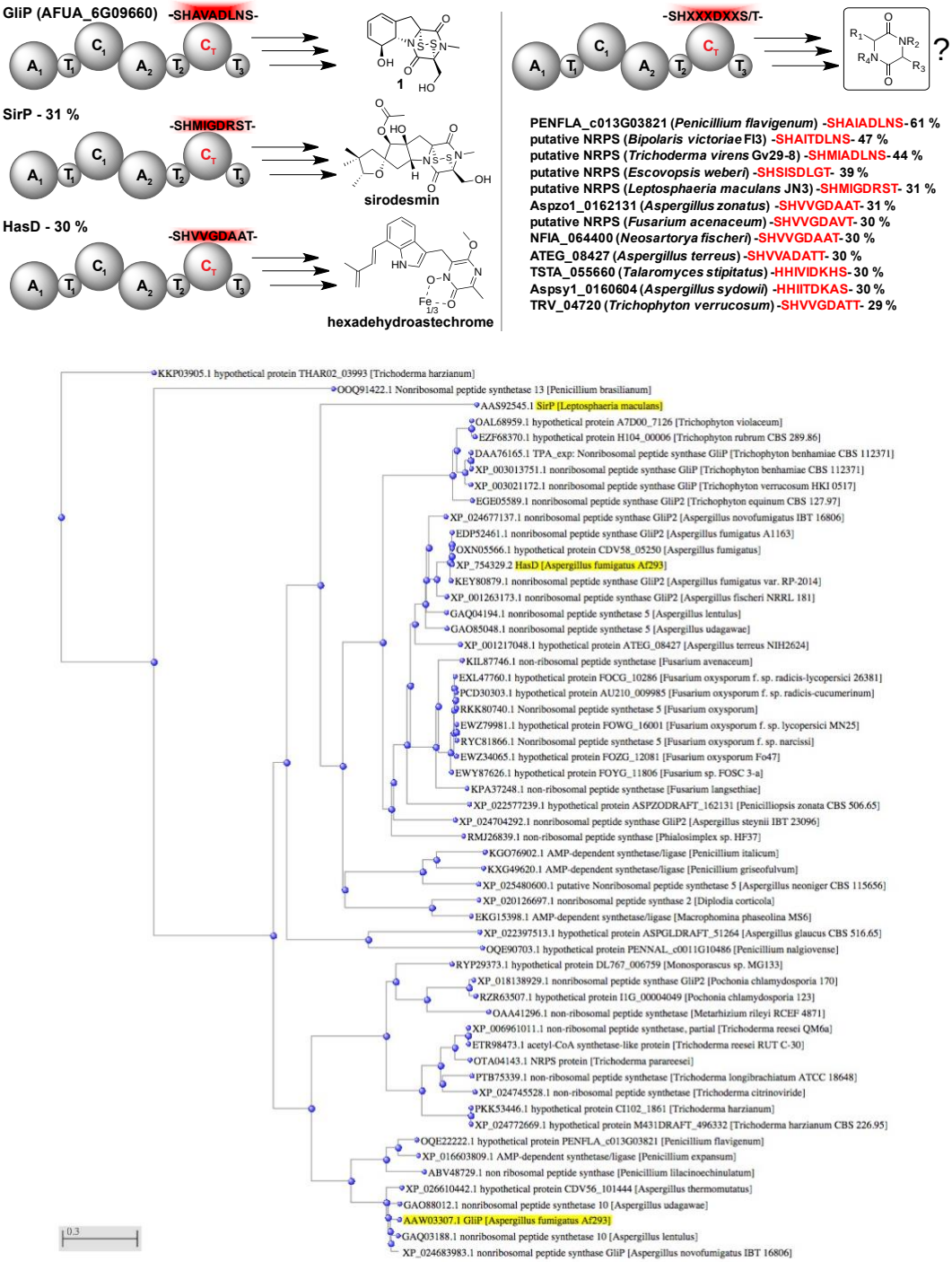


Figure S9: Examples for conservation of the C_T domain in confirmed and putative DKP producing fungal NRPSs. (top) Conserved amino acid sequence in the C_T domains are highlighted in red text. Percentages are total amino acid similarity. (bottom) Phylogenetic tree for GliP homologs containing C_TT_C tandem, see **Supporting Methods 1.13** and **Table S1** for more information. Scale bar is Grishin distance.[16-18]

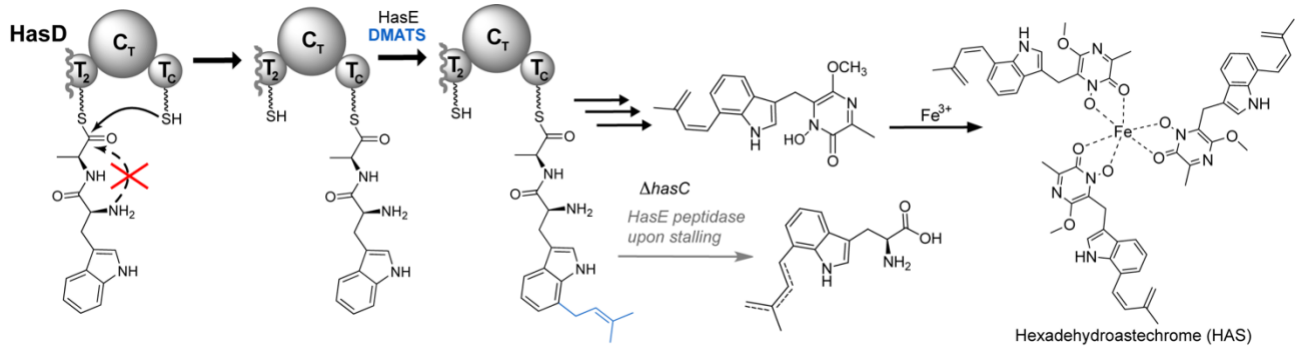


Figure S10: Model for hexadehydroastechrome biosynthesis in *Aspergillus fumigatus*. Prenylation of a T_C-tethered dipeptide (as opposed to prenylation of the cyclized DKP) would explain copious production of prenyltyptophan in Δ*hasC* mutant background (see references 17 and 19). DMATS: dimethylallyltryptophan synthase.[17,19]

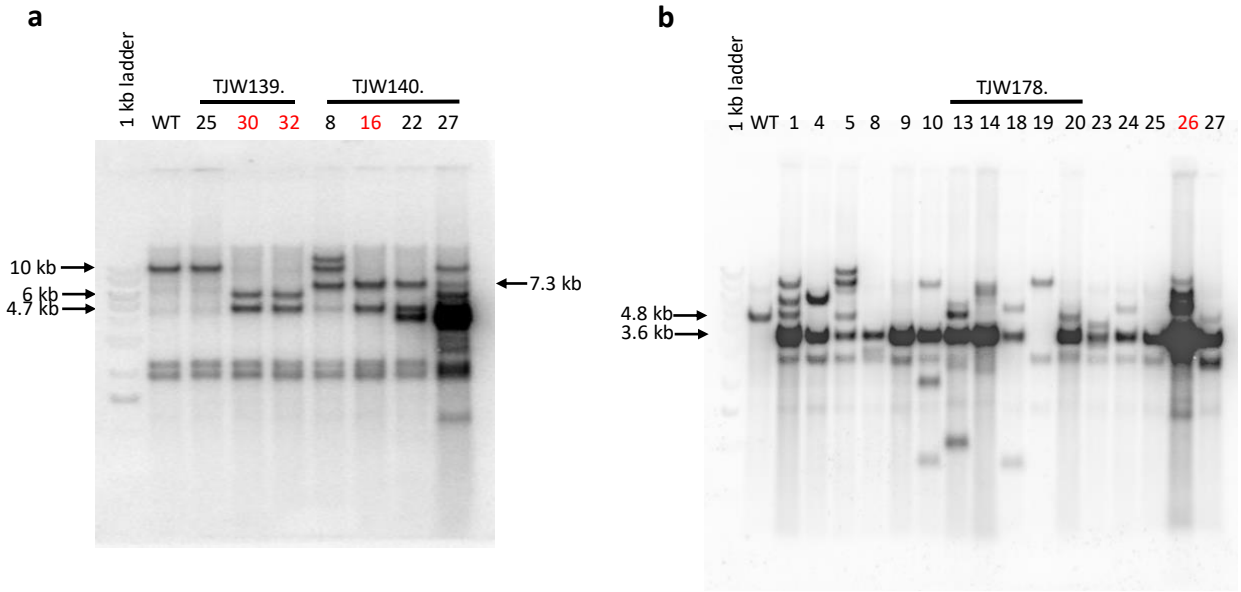


Figure S11: Southern confirmation. (a) *A. fumigatus* C_TT_3 and T_3 deletion mutants. Genomic DNA was digested by *Nde*I. WT (10 kb), C_TT_3 deletion (4.7 and 6 kb) and T_3 deletion (4.7 and 7.3 kb). TJW139.30 and TJW140.16 were chosen for the subsequent experiments. (b) Complementation of C_TT_3 deletion mutant. Genomic DNA was digested by *BamH*I and *Hind*III with 3.6 kb fragment expectation. TJW178.26 was chosen for subsequent experiment.

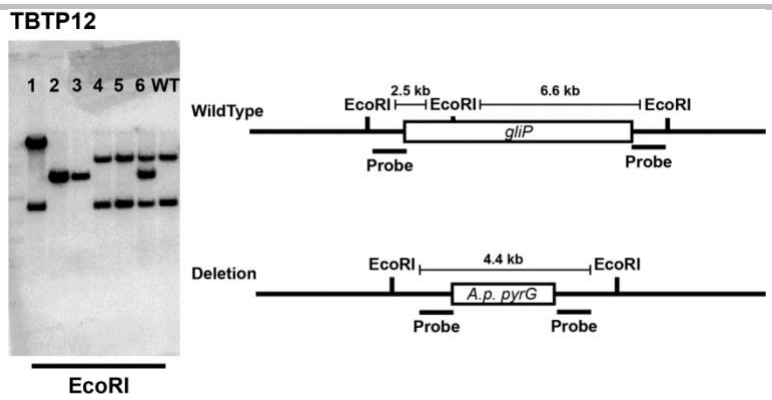


Figure S12: Confirmation of *gliP* deletion strain. The GliP open reading frame was replaced with a copy of *pyrG* from *A. parasiticus*. Genomic DNA was digested by *EcoRI*; the wildtype (WT) parental control shows the expected bands of 6.6 and 2.5kb, and transformants 2 and 3 show the expected band size of 4.4kb.

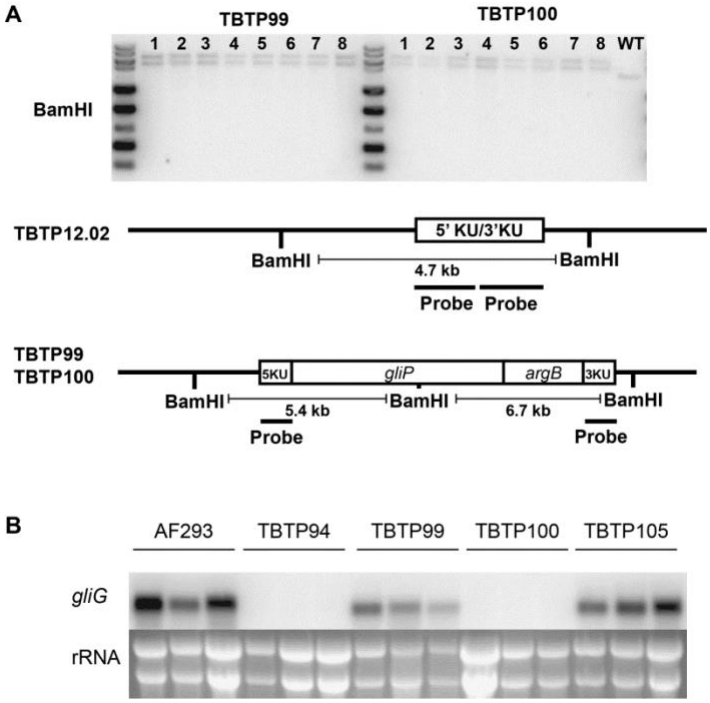


Figure S13: Confirmation of *gliP* complementation and cluster expression. (a) Genomic DNA was extracted and digested with *BamHI*; wild-type (WT) parental control shows the expected band of 4.7 kb while all transformants show the expected banding pattern of 6.7 and 5.4 kb. (b) Northern analysis of *gliG* expression in wild-type strains (AF293 & TBTP105) as well as Δ*gliP* (TBTP94), *gliP* complement strain (TBTP99) and the *gliP*-H1754A point mutant (TBTP100). Strains were grown in liquid GMM for 72 h at 25 °C at 225 RPM.

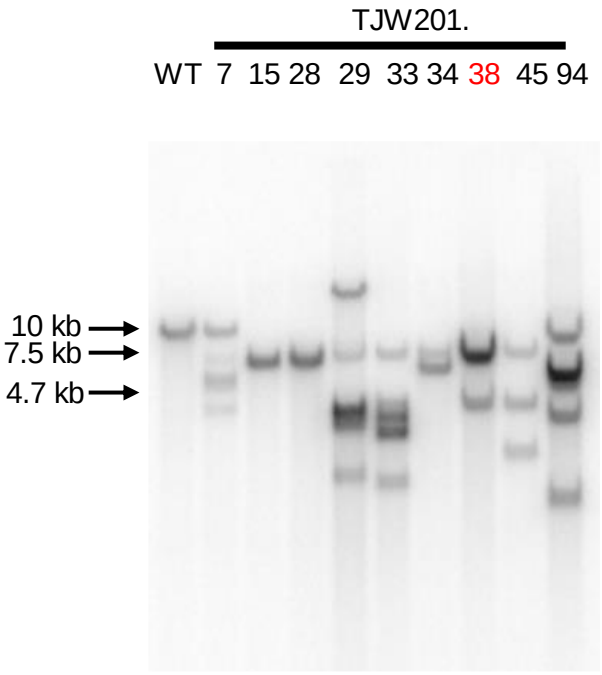


Figure S14: Southern confirmation of S2095A point mutant. Genomic DNA was digested by *Nde*I. WT (10 kb), and point mutation (4.7 and 7.5 kb). TJW201.38 was chosen for the subsequent experiments.

3 Supporting Tables

Table S1. Fungal NRPSs with homologous domain architecture to GliP

Accession	Name	Species	[%] similarity
GAQ03188.1	nonribosomal peptide synthetase 10	*Aspergillus lentulus*	93.192
XP_024683983.1	nonribosomal peptide synthase GliP	*Aspergillus novofumigatus IBT 16806*	91.589
GAO88012.1	nonribosomal peptide synthetase 10	*Aspergillus udagawae*	88.832
XP_026610442.1	hypothetical protein CDV56_101444	*Aspergillus thermomutatus*	87.482
XP_016603809.1	AMP-dependent synthetase/ligase	*Penicillium expansum*	60.784
OQE22222.1	hypothetical protein PENFLA_c013G03821	*Penicillium flavigenum*	60.492

ABV48729.1	non ribosomal peptide synthase	*Penicillium lilacinoechinulatum*	59.605
RYP29373.1	hypothetical protein DL767_006759	*Monosporascus sp. MG133*	40.071
ETR98473.1	acetyl-CoA synthetase-like protein	*Trichoderma reesei RUT C-30*	39.666
XP_006961011.1	non-ribosomal peptide synthetase, partial	*Trichoderma reesei QM6a*	39.666
OTA04143.1	NRPS protein	*Trichoderma parareesei*	38.861
PTB75339.1	non-ribosomal peptide synthetase	*Trichoderma longibrachiatum ATCC 18648*	38.482
PKK53446.1	hypothetical protein CI102_1861	*Trichoderma harzianum*	38.28
XP_024772669.1	hypothetical protein M431DRAFT_496332	*Trichoderma harzianum CBS 226.95*	38.28
XP_024745528.1	non-ribosomal peptide synthetase	*Trichoderma citrinoviride*	38.279
XP_018138929.1	nonribosomal peptide synthase GliP2	*Pochonia chlamydosporia 170*	37.562
RZR63507.1	hypothetical protein I1G_00004049	*Pochonia chlamydosporia 123*	37.466
OAA41296.1	non-ribosomal peptide synthetase	*Metarhizium rileyi RCEF 4871*	36.233
XP_022397513.1	hypothetical protein ASPGLDRAFT_51264	*Aspergillus glaucus CBS 516.65*	31.302
AAS92545.1	SirP	*Leptosphaeria maculans*	31.2
XP_022577239.1	hypothetical protein ASPZODRAFT_162131	*Penicilliopsis zonata CBS 506.65*	30.66
KGO76902.1	AMP-dependent synthetase/ligase	*Penicillium italicum*	30.45
KPA37248.1	non-ribosomal peptide synthetase	*Fusarium langsethiae*	30.277
XP_001263173.1	nonribosomal peptide synthase GliP2	*Aspergillus fischeri NRRL 181*	30.072
GAO85048.1	nonribosomal peptide synthetase 5	*Aspergillus udagawae*	30.072
XP_025480600.1	putative Nonribosomal peptide synthetase 5	*Aspergillus neoniger CBS 115656*	30.062
OQE90703.1	hypothetical protein PENNAL_c0011G10486	*Penicillium nalgiovense*	30
KEY80879.1	nonribosomal peptide synthase GliP2	*Aspergillus fumigatus var. RP-2014*	29.991
OXN05566.1	hypothetical protein CDV58_05250	*Aspergillus fumigatus*	29.95
EDP52461.1	nonribosomal peptide synthase GliP2	*Aspergillus fumigatus A1163*	29.937
KMK60067.1	nonribosomal peptide synthase GliP-like protein	*Aspergillus fumigatus Z5*	29.905
EWZ79981.1	hypothetical protein FOWG_16001	*Fusarium oxysporum f. sp. lycopersici MN25*	29.883

RYC81866.1	Nonribosomal peptide synthetase 5	*Fusarium oxysporum f. sp. narcissi*	29.883
GAQ04194.1	nonribosomal peptide synthetase 5	*Aspergillus lentulus*	29.878
XP_754329.2	HasD	*Aspergillus fumigatus Af293*	29.86
EWY87626.1	hypothetical protein FOYG_11806	*Fusarium sp. FOSC 3-a*	29.835
EKG15398.1	AMP-dependent synthetase/ligase	*Macrophomina phaseolina MS6*	29.786
EWZ34065.1	hypothetical protein FOZG_12081	*Fusarium oxysporum Fo47*	29.758
PCD30303.1	hypothetical protein AU210_009985	*Fusarium oxysporum f. sp. radicis-cucumerinum*	29.713
EXL47760.1	hypothetical protein FOCG_10286	*Fusarium oxysporum f. sp. radicis-lycopersici 26381*	29.668
RKK80740.1	Nonribosomal peptide synthetase 5	*Fusarium oxysporum*	29.668
XP_020126697.1	nonribosomal peptide synthase 2	*Diplodia corticola*	29.51
KIL87746.1	non-ribosomal peptide synthetase	*Fusarium avenaceum*	29.499
XP_001217048.1	hypothetical protein ATEG_08427	*Aspergillus terreus NIH2624*	29.451
RMJ26839.1	non-ribosomal peptide synthase	*Phialosimplex sp. HF37*	29.341
KXG49620.1	AMP-dependent synthetase/ligase	*Penicillium griseofulvum*	29.328
OAL68959.1	hypothetical protein A7D00_7126	*Trichophyton violaceum*	29.255
DAA76165.1	TPA_exp: Nonribosomal peptide synthase GliP	*Trichophyton benhamiae CBS 112371*	29.25
XP_003021172.1	nonribosomal peptide synthase GliP	*Trichophyton verrucosum HKI 0517*	29.185
XP_003013751.1	nonribosomal peptide synthase GliP	*Trichophyton benhamiae CBS 112371*	29.061
XP_024677137.1	nonribosomal peptide synthase GliP2	*Aspergillus novofumigatus IBT 16806*	29.04
EGE05589.1	nonribosomal peptide synthase GliP2	*Trichophyton equinum CBS 127.97*	28.799
EZF68370.1	hypothetical protein H104_00006	*Trichophyton rubrum CBS 289.86*	28.798
XP_024704292.1	nonribosomal peptide synthase GliP2	*Aspergillus steynii IBT 23096*	28.444
KKP03905.1	hypothetical protein THAR02_03993	*Trichoderma harzianum*	24.906
OOQ91422.1	nonribosomal peptide synthetase 13	*Penicillium brasilianum*	24.415

-NRPSs with >95 % similarity to GliP are excluded from this table, as they likely produce gliotoxin (**1**).

SUPPORTING INFORMATION

Table S2. LC-HRMS data of reported compounds

Compound	HR-ESI(+/-) Observed (m/z)	Ion	Calculated Ion Formula	Calculated m/z	Retention time [min]
1	263.1030	$[M\text{-}S_2+H]^+$	$C_{13}H_{15}N_2O_4^+$	263.1032	5.00
2	235.1080	$[M + H]^+$	$C_{12}H_{15}N_2O_3^+$	235.1082	2.30
3	279.0801	$[M\text{-}SCH_3+H]^+$	$C_{13}H_{16}N_2O_3S^+$	279.0803	3.84
4	309.0906	$[M + H]^+$	$C_{14}H_{18}N_2O_4S^+$	309.0908	5.00

Table S3. Fungal strains used in this study

Name	Genotype	Reference
Af293	Wild type	[20]
Af293.1	*A. fumigatus pyrG1*	[20]
Af293.6	*A. fumigatus pyrG1, argB1*	[20]
ARC2	Δ*gliP::para pyrG1*	[21]
TJW139.3	ΔC_2T_3 *gliP:: para pyrG1*	This study
TJW140.16	ΔT_3 *gliP:: para pyrG1*	This study
TJW201.38	*GliP2095$^{ser->ala}$::AfpyrG; pyrG1*	This study
TBTP94.1	*pyrG1*; Δ*gliP*::A.p *pyrG1*; Δ*akuA*	This Study
TBTP99.1	*pyrG1*; Δ*gliP*::A.p *pyrG1*; *gliP*::Af. argB::*akuA*	This Study
TBTP100.6	*pyrG1*; Δ*gliP*::A.p *pyrG1*; *gliP*-H1754A::Af. *argB*::*akuA*	This Study
TFYL44	*pyrG*$^-$; *argB*-; Δ*akuA*	[22]
TBPT12	*pyrG*$^-$; *argB*-; Δ*gliP*::A.p *pyrG*;Δ*akuA*	This study
TBTP94	*pyrG*$^-$; Δ*gliP*::A.p *pyrG*; Δ*akuA*	This study
TBTP99	*pyrG*$^-$; Δ*gliP*::A.p *pyrG*; *gliP*::Af. *argB*::*akuA*	This study
TBTP100	*pyrG*$^-$; Δ*gliP*::A.p *pyrG*; *gliP* H1754A::Af. *argB*::*akuA*	This study
TBTP105	Δ*akuA*::A.p. *pyrG*; *pyrG1*	This study

Table S4. PCR primer sets used in this study

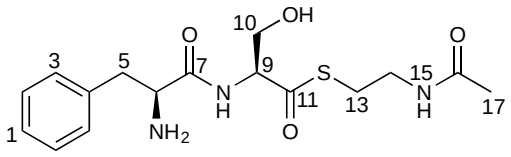
Name	Sequence (5'-3')	Purpose
DgPCT5'F	TGGTCTATGCAACAGGAGAACCC	$\Delta C_T T_3$
DgPCT5'R	GGGTGAAGAGCATTGTTTGAGGCGACCGGTTCAAAACG CGTCGCAGATGGTCCGTGGC	$\Delta C_T T_3$
glutapyrGF	TGAACCGGTCGCCTCAAACAATGC	$\Delta C_T T_3$
glutapyrGR	CTGTCTGAGAGGAGGCACTGATG	$\Delta C_T T_3$
DgpCT3'F	GGCATCACGCATCAGTGCCTCCTCTCAGACAGTTCTTCC ACACGGTATACATTGTAGCC	$\Delta C_T T_3$
DgPCT3'R	ATTCGCGAGCTCAACCGCATGG	$\Delta C_T T_3$
DgPT5'F	TTGTCGAGACATCACTCGGACC	ΔT_3
DgPT5'R	GGGTGAAGAGCATTGTTTGAGGCGACCGGTTCAGCAGG GGAACTCATCCAGCGAGACGCC	ΔT_3
gliPgpdF	AAAGTCACAGGATCCAAGCTGTAAGGATTTCGGCACGG	$\Delta C_T T_3$ complementation
glipgpdR	GCGTGGAGTGCTCGATCACATCGCGCATTGTGATGTCTG CTCAAGCGGGGTAGCTG	$\Delta C_T T_3$ complementation
CTgliPF	CAGCTACCCCGCTTGAGCAGACATCACAATGCGCGATGT GATCGAGCACTCCACGC	$\Delta C_T T_3$ complementation
CTglipR	CCATGTCAAAGCTTATATCATCTACGCTGGGACGCG	$\Delta C_T T_3$ complementation
gliP 3'-F	GATAGCACACCCTCGGAATAGTCCTCTCGGCGTTCCATT CGACAGAAGACGAGG	Deletion
gliP 3'-R	CTGGAGCAGCTTCCGTGC	Deletion
gliP 5'-F	GAGGCTCTGCTCAGATGAGG	Deletion
gliP 5'-R	CGATGATAAGCTGTCAAACATGAGGCAGAGCGTAGGGTT GAGC	Deletion
Ap-pyrG-F	CTCATGTTTGACAGCTTATCATCG	Deletion
Ap-pyrG-R	CCGAGAGGACTATTCCGAGG	Deletion
YS F	TTCGCGTACTGACAGCACAGG	Complement
YS R	GCAAGACGGCGAGACTGTTCC	Complement
KU5'-F	TATTGCCGTTGGATCTTTGGGG	Complement

KU5'-R	GGTATGGATTGTCATCAGCCATAGTGAG	Complement
gliP-F	TTCTCACTATGGCTGATGACAATCCATACCCGCTCGCCAATATGCTTGC	Complement
gliP-R	GAAAATTTGTCTTGGATGCAGACCGCGTTCCTGTGACGAACTCGACGAGG	Complement
gliP-H1754A-R	ACGGCTGCGCTGGTAACCACAAGGAGG	Complement Mutant Copy
gliP-H1754A-F	GCACCCTCCTTGTGGTTACCAGCGCAGCCGTCGCCGATCTCAACAGCGTG	Complement Mutant Copy
AFU argB fwd	GAACGCGGTCTGCATCCAAG	Complement
AFU argB rev	TGGTTAGTAACATTCAGACAGTCGGCATGCAGGGACTGAACCTGGTGAATCG	Complement
KU3'-F	GCATGCCGACTGTCTGAATGTTACTAACC	Complement
KU3'-R	TCACATGTTCTTTCCTGCGTTATCCCCTACACCAAGAAGCTCACCACCCC	Complement
AFU argB rev2	AGCATCCATTCTGCGTCTCG	Complement
gliP_S555A_fwd	AAGAGCAGCAAGGGCGTTTCCGCCCATGG	SDM of gliP-S555A
gliP_S555A_rev	CCATGGGCGGAAACGCCCTTGCTGCTCTT	SDM of gliP-S555A
gliP_H1754A_fwd	TTGTGGTTACCAGCGCCGCCGTCGCCGATC	SDM of gliP-H1754A
gliP_H1754A_rev	GATCGGCGACGGCGGCGCTGGTAACCACAA	SDM of gliP-H1754A
gliP_S1582A_rev	CTGGAGAACGGCATGGCCGCCCAGAGC	SDM of gliP-S1582A
gliP_S1582A_rev	GCTCTGGGCGGCCATGCCGTTCTCCAG	SDM of gliP-S1582A
pET21_gliPC_TT_3_fwd	CtctagaaataattttgtttaactttaagaaggagatatacatATGCGCGATGTGATCGA	Truncation to express gliPC2T3
pETet21_gliPC2T3_rev	tgttagcagccggatctcagtggtggtggtggtggtgAAGAACCTGACGGTAAAGACGCT	Truncation to express gliPC_TT_3
Ptmt5'F	AGTCATTCAACGCCGTGTTGGC	S2095A mutation

Ptmt5‘R	GACGGCTCAACCGCCAGGCCTGGATCGCGTCGCCGCC GGCCTCGAAGAAGGAG	S2095A mutation
Ptmt3‘F	GGCGACGCGATCCAGGCCTGGCGGTTGAGCC	S2095A mutation
Ptmtnested5‘R	GGTGAAGAGCATTGTTTGAGGCGACCGGTTCAAAGA ACCTGACGGTAAAGACGCTGTGCC	S2095A mutation
PtmtglutpyrGF	CGTCAGGTTCTTTGAACCGGTCGCCTCAAACAATGC	S2095A mutation
gliGF	AAAGGTGAGTCGAGTCGACGC	Northern probe
gliGR	ATACTCTTTCTCGCCATGGCC	Northern probe
gliIinF	TTCGTTGGCACCGCATGCATGG	Northern probe
gliIinR	AGATAGCCGTCCATTTCTGCCC	Northern probe
gliJF	AAGAGGTACCTCTGATCGACGG	Northern probe
gliJR	TATCCTCGTTCCACACCTCGTCG	Northern probe
gliCF	AGTTCTTCCGCAACTCGCACC	Northern probe
gliCR	AGCCAGGAATGTGTCATCCCG	Northern probe

Table S5. ^{1}H (600 MHz) and ^{13}C (151 MHz) NMR spectroscopic data for compound **5** in a 80:20 mixture of methanol-d_4 : chloroform-d_3.

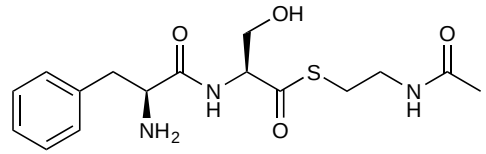
Chemical shifts were referenced to δ(C<u>H</u>D$_2$OD) = 3.31 ppm and δ(^{13}<u>C</u>HD$_2$OD) = 49.00.^{13}C chemical shifts were determined via HMBC, HSQC and direct observation ^{13}C spectra. ^{1}H, ^{1}H-*J*-coupling constants were determined from the acquired ^{1}H or dqfCOSY spectra. HMBC correlations are from the proton(s) stated to the indicated ^{13}C atom.



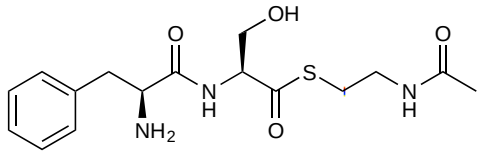
5

No.	δ_c	Proton	δH(J_{HH}[Hz])	HMBC
1	129.28	1-H	7.24 ($J_{1,2}$ = 7.5)	3
2	127.68	2-H_2	7.21 ($J_{2,1}$ = 7.5) ($J_{2,3}$ = 7.4)	2,4
3	128.92	3-H_2	7.25 ($J_{3,2}$ = 7.4) ($J_{3,5}$ = 1.0)	1,3,5
4	133.65			
5	36.87	5-H_a	2.98 ($J_{5a,5b}$ = 14.0) ($J_{5a,6}$ = 8.2) ($J_{5a,3}$ = 1.0)	3,4,6,7
		5-H_b	3.28 ($J_{5b,5a}$ = 14.0) ($J_{5b,6}$ = 5.5) ($J_{5a,3}$ = 1.0)	3,4,6,7
6	54.39	6-H	4.26 ($J_{6,5a}$ = 8.2) ($J_{6,5a}$ = 5.5)	4,5,7
7	168.86			
8		8-NH		
9	61.54	9-H	4.55 ($J_{9,10a}$ = 4.0) ($J_{9,10b}$ = 4.5)	7,10,11
10	61.70	10-H_a	3.75 ($J_{10a,10b}$ = 11.8) ($J_{10a,9}$ = 4.0)	9,11
		10-H_b	3.84 ($J_{10b,10a}$ = 11.8) ($J_{10b,9}$ = 4.5)	9,11
11	198.19			
12				
13	28.44	13-H_2	2.90 ($J_{13,14}$ = 12.0)	11,14
14	38.48	15-H_2	3.27 ($J_{14,13}$ = 12.0)	13,16
15		15-NH		
16	172.01			
17	22.31	17-H_3	1.86	16

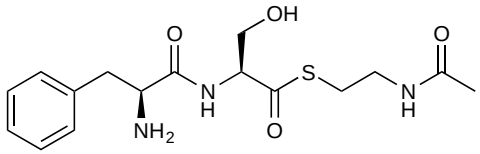
^{1}H NMR spectrum ($CD_3OD/CDCl_3$, 600 MHz) of **5**



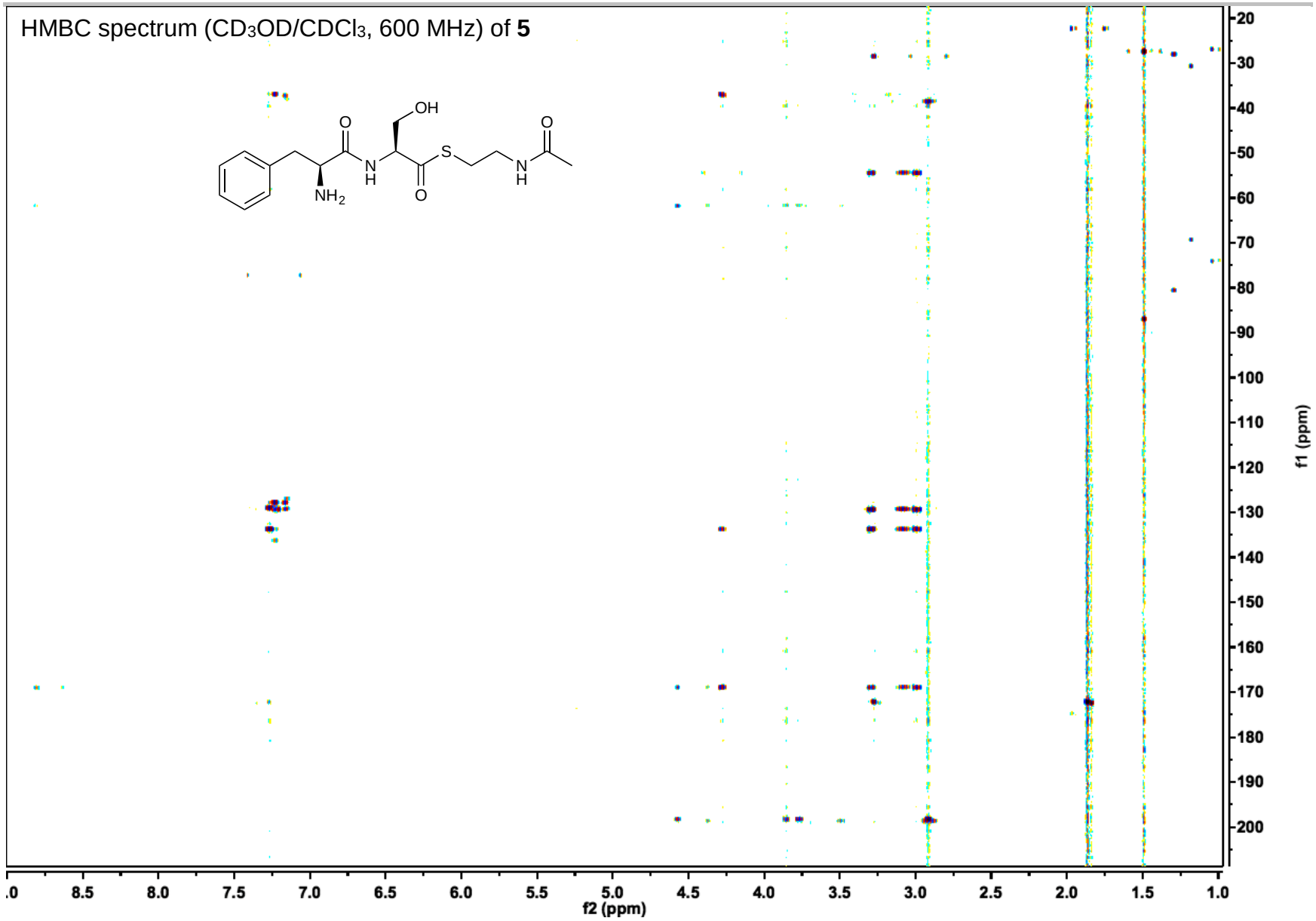
dqfCOSY spectrum ($CD_3OD/CDCl_3$, 600 MHz) of **5**



HSQC spectrum ($CD_3OD/CDCl_3$, 600 MHz) of **5**



HMBC spectrum ($CD_3OD/CDCl_3$, 600 MHz) of **5**



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